Impact of Halogenated Compounds on Calcium Homeostasis in Hepatocytes

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Halocarbons (CCl₄, 1,1-dichlorethylene) cause a wide spectrum of effects and injury in hepatocytes. One early effect of these compounds is the inhibition and destruction of the endoplasmic reticulum (ER) calcium pump. Subsequent to inhibition of this pump, the ER calcium pool is depleted and cytosolic levels of calcium are increased for a prolonged period of time. This effect of halocarbons has been characterized and is similar *in vivo* and *in vitro*. The importance of this redistribution of cell calcium in expression of halocarbon injury of hepatocytes has not been fully resolved. Several degradative enzymes (phospholipases, proteases) have been implicated as calcium-dependent mediators in toxicity. Our preliminary studies of the effect of calcium redistribution suggest that activation of a calcium-sensitive endonuclease in liver does not play a central role in initiating the lethal effect of halocarbons on hepatocytes.

Introduction

Cytosolic calcium is an important regulator of the activity of many metabolic and structural proteins. Cells normally maintain cytoplasmic calcium at very low levels. Calcium concentrations briefly rise severalfold in response to physiological stimuli (1). The potential role of disrupted calcium flux in chemically induced liver injury have been examined (2–6). Recently, attention has turned to examining early alterations of intracellular calcium homeostasis as causative in halocarbon-induced lethal injury (7–9).

The low resting concentration of ionized calcium in cytosol is maintained by active compartmentation processes. The level of ionized calcium in cytosol is determined by the cooperation of at least three compartmentation processes—the plasma membrane Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase, the endoplasmic reticulum (ER) Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase, and the mitochondrial Ca^{2+}/H^+ antiporter. Microsomal fractions isolated from a number of nonmuscle cells and tissues have an energy-dependent calcium uptake system (10–12). The calcium pump in this fraction is thought to principally represent activity of the endoplasmic reticulum (ER). Several investigators have noted similarities between these calcium pumps and the skeletal muscle sarcoplasmic reticulum calcium pump. These similarities suggest that the ER (microsomal) calcium pump functions to sequester cytoplasmic calcium and, thus, participates in regulating ionized calcium in the cytoplasm and maintaining a releasable, intracellular pool of calcium.

Reynolds and co-workers (13) first observed a selective loss of calcium from the endoplasmic reticulum of rat liver after administrations of CCl₄. Subsequently, it was demonstrated that CCl₄ rapidly causes the inhibition of the ER calcium pump (14). This pump is thought to play an essential role in regulating cytoplasmic calcium and to provide an intracellular pool of calcium in the hepatocyte that can be released in response to exogenous stimuli. When this calcium pump is destroyed, a pool of calcium appears to be released from the ER (15). More recently, we have demonstrated that cytoplasmic concentrations of ionized calcium increase rapidly and for prolonged periods both *in vivo* (15) and *in vitro* (16) after an animal or hepatocytes are exposed to CCl₄ or 1,1-dichloroethylene.

The Calcium Hypothesis

The calcium hypothesis of acute cell injury suggests that something happens during toxic injury to overload the normal homeostatic mechanisms maintaining ionized cytosolic calcium in the nanomolar range. The overload could occur by the toxicant damaging

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one or more of the energy-dependent calcium seques-tration mechanisms, or by the toxicant allowing a massive influx of extracellular calcium. Halocarbons appear to inhibit or destroy either the ER or plasma membrane calcium pumps and in some cases to selec-tively increase membrane permeability to calcium.

A number of toxic events, including exposure to halocarbons, have been shown to elevate ionized cal-cium in cytoplasm to supraphysiological levels or for supraphysiological times. Currently, the element missing is demonstration of an event common to all toxicant models and resulting from calcium levels outside the normal range.

Effect of Halocarbons on Intracellular Calcium

Effect on the Endoplasmic Reticulum Pump and Cytosolic Calcium

Results from *in vivo* experiments are presented in Figure 1. Following a hepatotoxic, but not lethal, dose of CCl_4, the ER calcium pump activity declined to 35% of control by 30 min. Pump activity remained depressed throughout the experiment and was 6% of control by 8 hr. In livers from rats that received CCl_4, phosphorylase a activity was 133% of control by 30

min and 150% of control by 1 hr. At 8 hr hepatic phosphorylase a activity remained at 135% of control. Total phosphorylase ($a + b$ forms) activity did not vary significantly over 8 hr in controls or in CCl_4-treated rats (data not shown). Measurement of liver cAMP concentrations demonstrated no statistically significant differences at any time between the treated and control groups (*17*). These observations allow us to use phosphorylase a activity as a measure of ionized calcium in rat liver cytoplasm. The activity of another endoplasmic reticulum enzyme, glucose-6-phosphatase, activity was not significantly affected until 2 hr (71% of control). By 8 hr this enzyme activ-ity was approximately halved. In contrast, this enzyme is not inhibited after exposure of an animal to toxic doses of 1,1-dichloroethylene, a halocarbon that does not initiate lipid peroxidation (*18,19*). Activity of the plasma membrane enzyme 5'-nucleotidase was not effected by CCl_4 administration. To confirm the effect of CCl_4 on phosphorylase a levels, glycogen con-centrations in liver were examined. Liver glycogen content was reduced to 68% of control as early as 30 min, and glycogen concentrations declined progres-sively until 24 hr after CCl_4, when liver glycogen could not be detected. Serum levels of glutamic-pyruvic transaminase were elevated by 8 and 24 hr to 9-fold control. The accumulataion of total calcium by liver
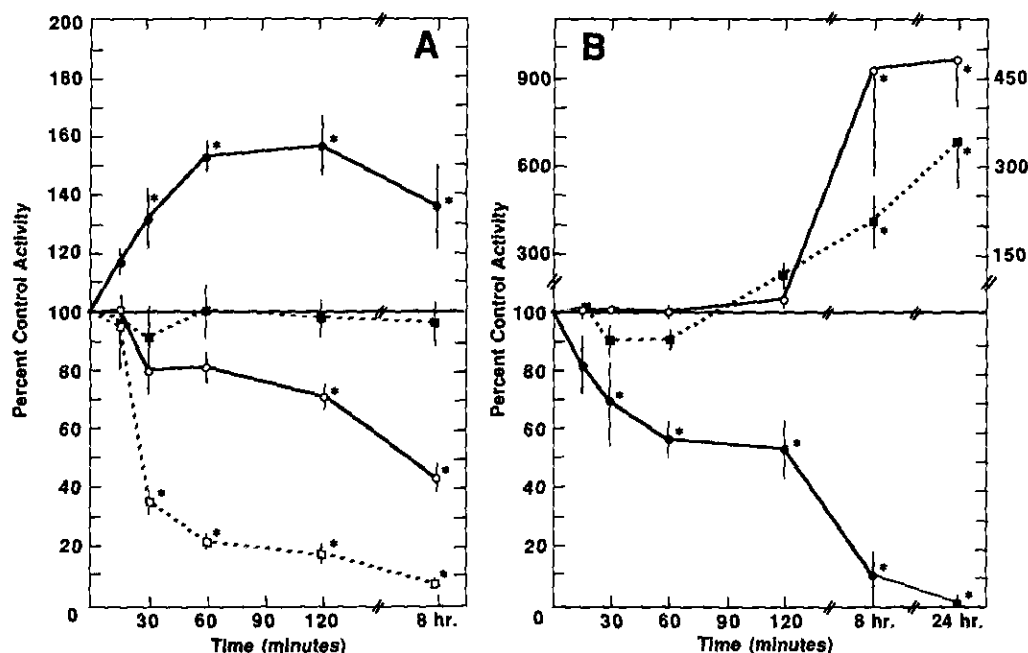

FIGURE 1. Time course of CCl_4-induced biochemical alterations *in vivo.* (*A*) Phosphorylase a (●), ER calcium pump (□), glucose-6-phosphates (○), and 5'-nucleotidase (■) activities in liver homogenates. (*B*) Glycogen (●), serum glutamic-pyruvic transaminase (○, top left scale), and liver calcium (■, top right scale) concentrations in liver or blood from the same animals. Rats were given CCl_4 (1.5 mL/kg) or vehicle at 0 min. Control and treated animals were killed at each time point ($n = 3$ to 5 for each group). Results are expressed as means ± SEM. In liver tissue sampled from control rats, ER calcium pump activity averaged 190 ± 11 nmole calcium/mg protein/30 min, phosphorylase a activity ranged from 44 ± 9.8 to 82 ± 11 nmole phosphate/mg protein/min at 8 AM and 5 PM, respectively: glucose-6-phosphatase activity averaged 1.59 ± 0.04 μmole phosphate/mg protein/20 min; 5'-nucleotidase averaged 0.98 ± 0.03 μmole/mg protein/20 min; glycogen content declined from 39 ± 3.4 mg/g liver to essentially zero by 24 hr because food was withheld from all animals after dosing; total liver calcium averaged 55.9 ± 1.6 μg/g liver; serum levels of glutamic-pyruvic transaminase averaged 19.6 ± 1.4 IU/L in control plasma. Differences are significant where indicated ($p \leq 0.05$) by ANOVA (*17*).
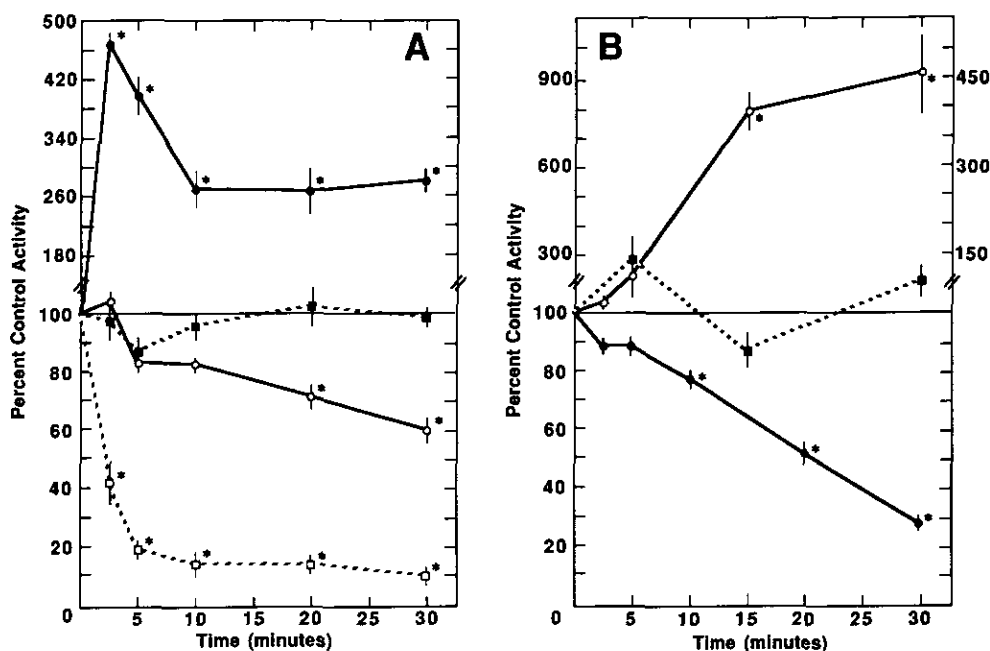
FIGURE 2. Time course of CCl₄-induced biochemical alterations *in vitro*. (*A*) Phosphorylase *a* (●), ER calcium pump (□), glucose-6-phosphatase (○), and 5′-nucleotidase (■) activities in cells or culture medium. (*B*) Glycogen (●), serum glutamic-pyruvic transaminase (○, top left scale), and hepatocyte calcium (■, top right scale) concentrations in cells or culture medium. Hepatocytes were exposed to CCl₄ (0.2 μL/mL) or vehicle at 0 min. Control and treated cell cultures were collected at each time point (*n* = 3 cell preparations). Results are expressed as means ± SEM. *In material sampled from control cultures, ER calcium pump activity averaged 61.8 ± 3.2 nmole calcium/mg protein/30 min; phosphorylase a activity ranged from 44.5 ± 2.3 nmole phosphate/mg protein/min; glucose-6-phosphatase activity averaged 1.07 ± 0.03 μmole phosphate/mg protein/20 min; 5′-nucleotidase averaged 0.98 ± 0.03 μmole/mg protein/20 min; glycogen content of control cells averaged 93.5 ± 3.6 μg/mg protein and did not vary throughout the experiment; hepatocyte total calcium averaged 191 ± 12 μg/mg protein in control cultures; glutamic-pyruvic transaminase was not present in the medium over control cultures in quantities ≥ 5% of the total cellular content. Differences are significant where indicated (p ≤ 0.05) by ANOVA (17).*

accompanied a loss of cytoplasmic enzymes. Liver calcium rose to 207 and 382% of control levels 8 and 24 hr after CCl₄.

Results from *in vitro* experiments are represented in Figure 2. Cultures of rat hepatocytes (18 hr old) were exposed to a toxic concentration of CCl₄. ER calcium pump activity was depressed to 41% of control at 2.5 min, 19% of control at 5 min and pump activity remained at 10% of control after 30 min. Phosphorylase *a* activity in cultured hepatocytes was stimulated to 472% of control at 2.5 min and to 392% of control at 5 min. Activity remained elevated beyond 30 min. Conversion of phosphorylase from the *b* to the *a* form was complete, as judged from measurement of total *a* + *b* activity (data not shown). Stimulation appeared to be greater than had been observed at early times *in vivo*; however, consistently low control phosphorylase *a* activity *in vitro* exaggerated this effect. Glucose-6-phosphatase activity was not significantly inhibited until 20 min after CCl₄ exposure. 5′-Nucleotidase activity was never significantly inhibited after CCl₄ exposure, as *in vivo*. Glycogen content of cells were depressed to 77% of control by 10 min. Glycogen continued to be mobilized at the same rate until it was 27% of control at 30 min. Later indices of liver cell injury were also examined in hepatocytes cultures. Glutamic-pyruvic transaminase release was massive by 15 and 30 min after

CCl₄ (10-fold greater than control). Hepatocyte calcium (total of ionized and bound) never increased in the *in vitro* experiments. Only in this determination did the *in vitro* model depart from results observed with liver tissue from the intact rat. (See below.)

Effect on the Plasma Membrane Pump

Hepatotoxic doses *in vivo* of CCl₄, bromobenzene, or acetaminophen produce inhibition of the liver plasma membrane calcium pump (20). After CCl₄, permeability of plasma membrane vesicles to calcium is substantially increased (21). In contrast, not all hepatotoxins inhibit the ER calcium pump; bromobenzene and acetaminophen inhibit only the hepatic plasma membrane calcium pump (20).

Effect on Mitochondrial Calcium Sequestration

In vivo mitochondrial calcium sequestration is not inhibited by CCl₄ (14), and mitochondria accumulate great quantities of calcium parallel to tissue death, thus increaseing the total calcium in the liver (13,14). In hepatocyte culture exposed to a range of CCl₄ concentrations, mitochondrial as well as ER calcium sequestration could be depressed (Fig. 3). The data presented in this figure suggest that, in our culture
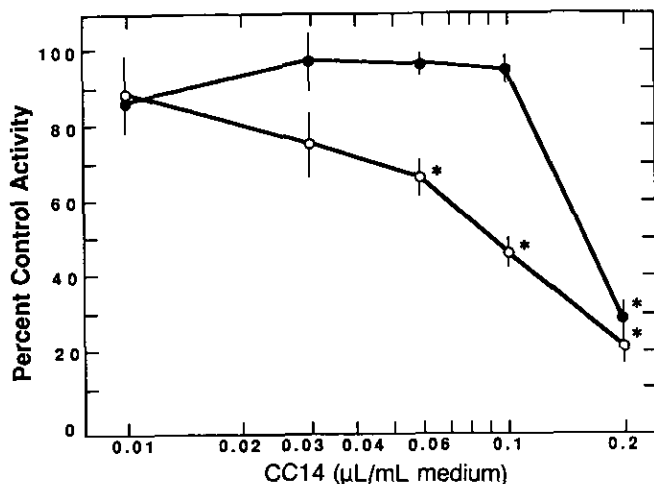
FIGURE 3. ER and mitochondrial calcium sequestration in material isolated from hepatocytes exposed to CCl_4. Hepatocyte cultures were exposed to CCl_4 (0.01 to 0.2 μL/mL or vehicle for 5 min). Cells were then homogenized and promptly assayed under conditions optimized for ^{45}Ca sequestration by ER (○) or mitochondria (●). Results are expressed as mean ± SEM. In control cultures, mitochondrial calcium sequestration averaged 58.4 ± 9.7 nmole calcium/mg protein/10 min and ER calcium pump averaged 94.1 ± 9.9 nmole calcium/mg protein/30 min. Differences are significant where indicated ($p \le 0.05$) by ANOVA (17).

system, exposure of hepatocytes to initial CCl_4 concentrations than 0.1 μL/mL do not completely model CCl_4 action *in vivo*. Inhibition of mitochondrial calcium uptake by CCl_4 in homogenates of cultured hepatocytes accounts for the lack of calcium accumulation by the injured cells.

Effect of Altered Calcium Homeostasis on Cell Function

The relationship between a sustained increase of cytosolic calcium and toxicity by a number of halocarbons has lead several groups to search for a mechanism or mechanisms by which an increase of calcium could initiate an event or events ultimately leading to death of the hepatocyte. It is assumed that a prolonged increase of ionized calcium to supraphysiological levels may cause a profound and prolonged stimulation of a normal (physiological) process that irreversibly alters the plasma membrane and destroys the semipermeable character of the plasma membrane.

Recently, several groups have examined whether or not phospholipase activation may contribute to the expression of toxicity in hepatocytes. Hydrolysis of phospholipids is increased after a toxicant insult (22–25). It is widely recognized that calcium plays some role in the control of intracellular proteolysis in several tissues. This system is best characterized in skeletal muscle (26). Activation of nonlysosomal proteases in hepatocytes has been demonstrated following exposure to a toxicant (27).

Effect of Increased Cytosolic Calcium on Endonuclease Activity

Endonucleases are among the hepatic enzymes having activity that can be stimulated by Ca^{2+} (28,29). Activation of endonucleases has been implicated in thymocyte killing produced by glucocorticoids both *in vivo* (30,31) and *in vitro* (32). We have examined whether or not liver endonuclease activity is stimulated by this increase in intracellular ionzed calcium and if such activation could play a role in initiating the ensuing hepatotoxic events.

Endonuclease activation was assessed by separating liver nuclear DNA on agarose gels to determine if DNA was fragmented. No generalized DNA fragmentation was observed *in vivo*, and in hepatocytes, fragmentation occurred only at very late times following the loss of plasma membrane integrity. Endonuclease activity was further examined at a more sensitive level by specifically monitoring serum albumin DNA. This gene has been shown to have characteristic cleavage sites, in the 5' flanking region, that are hypersensitive to endonuclease action. No changes were observed in the fragment sizes of DNA prepared from rat liver or hepatocyte DNA at early times when toxicity was developing. Thus, our evidence suggests no stimulation of endonuclease activity occurs within a time frame consistent with the rise of intracellular ionized calcium. We conclude that nucleases likely do not mediate the hepatotoxicity accompanying increased cytosolic calcium that follows halocarbon exposure.

Summary and Conclusions

A diversity of toxic insults, including thioacetamide, glactosamine, dimethylnitrosamine, heavy metals, and ischemia are known to alter tissue calcium content significantly. These agents may exert their effects initially by disrupting intracellular calcium homeostasis. Elevated cytosolic calcium concentrations that are supraphysiological, either in duration or in magnitude, may excessively activate a variety of calcium-dependent enzymes found at multiple locations within cells. Calcium-responsive degradative enzymes activated in an uncontrolled manner may damage sites distant (e.g., plasma membrane) from the original cellular injury. Potentially, this scheme (early redistribution of intracellular calcium, producing elevated ionized calcium in cytosol, which causes over-stimulation of calcium responsive enzymes) will prove to be a mechanism for initiating the irreversible development of cellular injury common to a wide variety of toxic substances and target tissues.

the views of the Department of Defense or USUHS. These experiments were conducted according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Animal Resources, National Research Council, DHEW Pub. No. (NIH) 78-23.

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